OSTEOPROTEGERIN IN MILK

Cross-Reference to Related Application

This application is a continuation of International application PCT/EP/02/02912 filed March 15, 2002, the entire content of which is expressly incorporated herein by reference thereto.

Background Art

The present invention pertains to osteoprotegerin obtainable from milk sources, and in particular, from human or bovine milk. The present invention also relates to the use of osteoprotegerin for preparing an ingestible preparation and/or a pharmaceutical composition, in particular for use in a method for preventing or treating disorders associated with bone metabolism or immune function.

In mammals, the bones provide support for the body and consist of minerals, a matrix of collagenous and non-collagenous proteins, and a cellular component. The growth and maintenance of such components are controlled by a variety of different factors involving regulation and interaction of its component cell types, i.e., the chondrocytes which form cartilage, the osteoblasts which synthesize and deposit bone matrix, and the osteoclasts responsible for resorption of bone material.

Chondrocytes are derived from mesenchymal cells and generate an initial cartilage template required for endochondral bone formation. Osteoblasts, which promote formation of bone tissue, are derived from mesenchymal osteoprogenitor cells and are located on the surface of bones where they synthesize, transport and arrange the matrix proteins. On the other hand, osteoclasts, which are responsible for bone resorption, are derived from granulocyte-monocyte precursors present in the hematopoietic marrow. The actions of osteoclasts and osteoblasts are tightly linked e.g., during the process of osteoclast mediated resorption, the protein factors which are elaborated act as signaling molecules to initiate bone renewal by osteoblasts. Osteoblasts, in turn, may influence osteoclast function through expression of soluble or membrane bound regulators. Normal bone remodeling is therefore dependent on a definite balance between the opposing functions of bone formation and bone resorption as conveyed by each of the respective cell types.

Growth factors such as fibroblast growth factor (FGF) and transforming growth factor (TGF)-β are stored in the bone extracellular matrix and when secreted stimulate the local

release of bone progenitor cells. Thereafter, factors such as bone morphogenetic proteins (BMPs) and parathyroid hormone (PTH) influence the development to these progenitors into osteoblasts, the bone-forming cells, whose final differentiation and function are regulated by the interaction of the cell with bone matrix proteins.

During ageing an individual is subject to a gradual loss in bone mass, a phenomenon termed uncoupling, which is deemed to result from the activity of osteoclasts exceeding that of osteoblasts. In cases where this uncoupling persists for a longer period of time, more and more of the bone's material gets destroyed/resorbed and a condition called osteoporosis results.

Apart from the age-dependent phenomenon, bone loss may also be brought about by calcium or hormonal deficiency or by conditions which result in a variety of different diseases such as osteoporosis, hypercalcemia, Paget's disease of bone, bone loss due to osteoarthritis or rheumatoid arthritis or osteomyelitis, and the like. The reduced bone density generally leads to a decreased mechanical strength and increased likelihood of fracture.

Current approaches for the treatment of osteoporosis and/or related bone disorders include the use of calcium administered to the individual in need thereof. Recently, agents involved in the stimulation and/or inhibition of bone cells, such as hormones, calcitonin, insulin-like growth factor or osteoprotegerin (OPG) have also been envisaged to be usable in treating the above disease conditions. These agents are generally prepared by recombinant means and have to be formulated/prepared in a galenic form such that the respective substance may reach the target, the bone, in an active form.

PCT publication WO 00/24771 discloses nucleic acids encoding osteoprotegerin like proteins and their use in e.g. the treatment of osteoporosis. The polypeptide is synthesized by recombinant means and then formulated to be compatible with the intended route of administration. Intravenous, intradermal, subcutaneous, oral (e.g. inhalation), transdermal (topical), transmucosal and rectal administrations can be used.

In general, it is quite time-consuming and cumbersome to find a suitable galenic form for a given substance, since the ingredients utilized for this purpose must be compatible with the active substance and must also provide sufficient protection against the different conditions in the body. However, since agents stimulating bone growth are synthesized locally – in or at the bone tissue - it is difficult to administer such a substance. Normally, capsules have to be devised which assist in passing the substance through the gastro-intestinal tract without getting destroyed by the adverse environmental conditions prevailing therein.

However, this route of administration also has some drawbacks since the substance has to pass the liver and be transported in body fluids before it reaches the bone. Furthermore, it often leads to a reduced amount of active biological material reaching the target tissue.

Consequently, this presents a problem as to how to provide a means of administering an active substance to an individual, whereby the substance acts in a specific target tissue in the individual. This problem now has been solved by the present invention.

Summary of the Invention

The present invention relates to a composition that provides osteoprotegerin obtainable from milk. The osteoprotegerin preferably has a glycosylation pattern giving rise to a polypeptide having a molecular weight of approximately 80, 130 and 200 kDa.

The osteoprotegerin can be provided in a composition. A typical composition may be a food material, an enteral composition or a pharmaceutical composition. In these materials and compositions, osteoprotegerin is present in an amount effective to assist in formation of lymphoid tissues and regulation of immune responses in a subject that consumes the composition.

The invention also relates to a method of making a food material or an enteral or pharmaceutical composition which comprises adding therein an amount of osteoprotegerin effective to assist in formation of lymphoid tissues and regulation of immune responses in a subject that consumes the composition.

Yet another embodiment relates to the ingestible product made by that method.

Another embodiment of the invention is a method for the treatment of disorders associated with bone remodeling or for the treatment or prophylaxis of immune disorders. These methods comprise administering to a person in need of such treatment one of the ingestible compositions of the invention. The immune disorder includes allergy, autoimmunity, inflammatory bowel diseases, systemic autoimmune conditions, dysregulation of cell proliferation and apoptosis and immunopathological conditions of the skin, the oral cavity, the gastrointestinal, urogenital or respiratory tracts. Also, the disorders may be associated with prematurity and/or low birth weight and the composition administered to a child in need of such treatment.

Brief Description of the Drawings

In the accompanying drawing figures,

Fig. 1 shows the concentration of osteoprotegerin in human breast milk during various stages of lactation;

Fig. 2 shows a Western blot analysis of human milk fractions under reducing conditions using 10% SDS-gel. Bands for OPG were revealed using the biotinylated anti-OPG polyclonal antibody, BAF805 from R&D Systems and streptavidin-alkaline phosphatase (SAPP);

Fig. 3 shows the restriction map of the plasmid which was integrated into the genomic DNA of *Yarrowia* transformants;

Fig. 4 shows a RT-PCR analysis of human breast milk cells and human mammary gland epithelial cells, MCF-7; Lanes 1 and 2 :β-actin (expected size band: 460 bp); Lanes 3 and 4 : OPG (expected size band: 603 bp); 1. Human breast milk cells; 2. MCF-7; 3. Human breast milk cells; 4. MCF-7;

Fig. 5 shows the results of an experiment, wherein the OPG of the present invention inhibits TRAIL-induced apoptosis of Jurkat cells.

Detailed Description of the Preferred Embodiments

Osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF) and TNF-receptor-like molecule 1 (TR1), is a recently described member of the tumor necrosis factor family of receptors (TNFR). It inhibits osteoclast development both in vitro and in vivo and increases bone density (osteopetrosis). In normal mouse embryos, OPG has been localized within cartilage rudiments of developing bones, as well as in the small intestine.

However, unlike other members of the TNF receptor family, OPG does not possess a transmembrane domain. Moreover, it could be shown that OPG is also a receptor for the cytotoxic ligand TRAIL (TNF-related apoptosis-inducing ligand) and is identical to follicular dendritic cell-derived receptor-1. As such, it is presumed to regulate cell death, as well as play an important role in the formation of lymphoid tissues and the regulation of immune responses. Indeed, animals lacking OPG have been shown to exhibit underdeveloped lymphoid tissues.

In the studies leading to the present invention, it has now surprisingly been found that in addition to its presence in e.g. the bone tissues, osteoprotegerin may also be found in human breast milk. In consequence, during breast feeding the mother is obviously supplying the newborn with this bioactive substance in a form that is capable of surviving in the child's gastro-intestinal tract. From this it follows that the OPG produced by mammary gland cells

obviously differs from OPG isolated from other sources as regards its stability and/or resistance to degradation.

Without wishing to be bound by theory, it is presently believed that the specific glycosylation pattern conveyed to the protein in mammary gland cells renders the polypeptide more stable vis-à-vis the acidic gastric fluid and/or the basic environment encountered in the intestine, so that upon intestinal absorption and transport to the bone tissue, the active domain remains intact and is capable of exerting its biological activity.

The OPG of the present invention, i.e. in a form obtainable from milk source, has a polypeptide sequence as identified by SEQ ID. No. 1 and exhibits sizes of about 80, 130 and 200 kDa, respectively, which differs from that obtained by recombinant means (i.e., 55 kDa).

The OPG of the present invention may be included in an ingestable preparation, which may be a food material, such as e.g. milk, yogurt, curd, cheese, fermented milks, milk-based fermented products, ice-creams, fermented cereal-based products, milk-based powders, infant formulae and also pet food. Likewise, the OPG of the present invention may also be included in a enteral or pharmaceutical composition e.g. selected from the group consisting of solutions, dried oral supplement, liquid oral supplement, dry tube feeding or liquid tube-feeding.

In fact, since the OPG according to the present invention is stable, there is no need to bring the active compound into a specific galenic form so as to protect it from the differing and potentially detrimental conditions prevailing in the gastro-intestinal tract and body fluids.

According to another aspect the present invention also provides for the use of osteoprotegerin from milk for preparing an ingestable preparation, such as a food material or an enteral composition, or a pharmaceutical composition.

The osteoprotegerin of the present invention and the ingestable preparation as detailed above may be used for the treatment and/or prophylaxis of disorders of bone remodeling.

The most common bone disorder is osteopenia, a condition relating in general to any decrease in bone mass to below normal levels. Such a condition may arise from a decrease in the rate of bone synthesis or an increase in the rate of bone destruction or both. The most common form of osteopenia is primary osteoporosis, also referred to as postmenopausal and senile osteoporosis. This form of osteoporosis is a consequence of an universal loss of bone with age and is usually a result of increase in bone resorption with a normal rate of bone formation. Yet other forms of osteoporosis include endocrine osteoporosis (hyperthyroidism, hyperparathyroidism, Cushing's syndrome, and acromegaly), hereditary and congenital forms

of osteoporosis (osteogenesis imperfecta, homocystinuria, Menkes' syndrome, and Riley-Day syndrome) and osteoporosis due to immobilization of extremities.

Quite recently, it has been acknowledged that osteoporosis in human populations has also been associated with a higher incidence of arterial calcification, a component of many atherosclerotic lesions.

Consequently, a food product as illustrated above may well be utilized for preventing the onset of or alleviating symptoms and/or structural changes in the bones associated with osteopenia or osteoporosis, respectively. It will be appreciated that the active substance will be included in the food material in an amount sufficient to effect a desired biological response. Since OPG has been found to be itself a constituent of mother's milk, milk-based products are inherently well suited for delivering the substance to an individual.

On the other hand, for treating severe cases of osteopenia or osteoporosis, respectively, the preferred regimen may be a pharmaceutical composition, which contains the osteoprotegerin according to the present invention in higher amounts, that is in amounts sufficient to stop or even revert the disease process. Such compositions may contain the OPG of the present invention as the only active substance. This has the advantage that no major formulation of the substance has to be envisaged. It is, therefore, well within the present invention to simply press a tablet consisting of "OPG-powder" optionally supplemented with carriers or flavoring agents. However, in the case that the OPG of the present invention shall be formulated together with other active substances, the nature and liability to degradation of these additional substances in the gastro-intestinal tract shall be considered. The OPG of the present invention formulated in dosage units, will enable the attending physician to more carefully control the daily or weekly dose of the active compound.

The osteoprotegerin of the present invention may also be utilized for preventing the onset of and/or treating Paget's disease of bone, osteomyelitis, infectious lesions in bone which lead to bone loss, hypercalcemia, osteonecrosis, bone loss due to osteoarthritis or rheumatoid arthritis, periodontal bone loss and/or osteolytic metastasis.

OPG has also been found to be a receptor for the tumor necrosis factor-related ligand (TRAIL) which induces apoptosis upon binding to its death domain-containing receptors. It is presumed to regulate cell death, as well as play an important role in the formation of lymphoid tissues and the regulation of immune responses. Furthermore, OPG is a decoy receptor for RANKL (ligand for the receptor activator of NF-κB) which has been reported as a product of activated T cells. Ligation of the receptor for RANKL on mature dendritic cells,

enhances dendritic cell survival. Furthermore, the engagement of RANKL with its receptor enhances T-cell growth and dendritic cell function.

Accordingly, the present invention provides for the use of osteoprotegerin obtainable from human and/or bovine milk for the manufacture of an ingestable preparation, such as e.g. a dietary composition or an enteral composition, or for the manufacture of a medicament, respectively, for contributing to the normal development of immune tissues, for contributing to normal immune function and even for preventing and/or treating disorders of the immune system.

Disorders of the immune system contemplated in the present invention comprise allergy, autoimmunity, sepsis, cancer, inflammatory bowel diseases, systemic autoimmune conditions, cardiovascular disease and immunopathological conditions of the skin, the oral cavity, the gastrointestinal, urogenital or respiratory tracts.

In addition, the osteoprotegerin of the present invention may likewise by applied for the regulation of cell proliferation and apoptosis, for the promotion of oral tolerance, the modulation of infectious processes and bacterial colonization of the neonate. Especially for neonates the above mentioned disorders may by and large be associated with prematurity and/or low birth weight, so that in these cases the osteoprotegerin of the present invention may simply be administered to the baby by means of baby food.

It will be appreciated that an individual at any age may be the individual to be treated, though babies and elderly are the main subjects to be considered due to their inherent requirement of exogenous osteoprotegerin. In particular individuals, such as newborns, require osteoprotegerin for the development of bone material and/or the immune system, so that in these cases the compound and/or the food material and/or the pharmaceutical composition of the present invention may be advantageously be administered.

However, it will be appreciated that the present invention may also be applied to adults, in order to prevent the onset of any of the above disorders. It will also be appreciated that apart from humans the individuals to be treated may also be animals, such as pets, in that the OPG of the present invention is included in pet food.

The OPG of the present invention may be obtained from a milk source, derived from a mammal, in particular from human or bovine milk or colostrum. Human milk OPG has an amino acid sequence of 380 aa and exhibits a molecular weight of approximately 80, 130 and 200 kda when compared to protein markers which were used as molecular weight standards

(BioRad). It exhibits 4 sites for N-glycosylation and may be present in a monomeric form and a dimeric form by forming a S-S bond via Cys^{379}.

The OPG of the present invention may be isolated from milk sources, such as human or bovine milk. However, it will be appreciated that the present OPG may be prepared by recombinant means in appropriate cells yielding a glycoslyation pattern as found in the "milk-OPG". Preferred cells for expression are those of the mammary gland, since these cells may be expected to yield an identical or essentially identical glycosylation pattern.

Suitable cells for expressing the present OPG may be obtained by immortalization with appropriate means, such as the SV40 vector or the telomerase gene, and transformation with an expression vector containing a nucleotide sequence encoding the OPG polypeptide. The polypeptide of interest may be obtained by isolating it from the supernatant, in the event that the polypeptide is secreted, or by collecting the cells and isolating the polypeptide from the cells themselves. In the event of a continuous production, isolation from the supernatant will be preferred.

Examples

The following examples illustrate the invention without limiting it thereto.

Human milk and human serum samples

Human breast milk samples (10-60 ml) from healthy mothers were collected up to 17 days post-partum under sterile conditions by breast pump expression or occasionally by manual expression. The milk was expressed into sterile 50 ml centrifugation tubes and processed within 2 hrs of collection. Following centrifugation (200 x g, 10 min), the cellular pellet was immediately removed and treated for RNA extraction. The remainder of the milk was frozen at –20°C. Human serum samples were obtained from healthy donors and kept at -20°C.

Fractionation of human breast milk

Cream was extracted from whole milk by high speed centrifugation. The top cream layer was removed, washed in water and the cream washings were frozen at –20°C until required. The separation of whey and casein was achieved by rennet enzyme treatment or chemical acidification (with HCl) of skimmed milk inducing casein clotting. Centrifugation

of the treated milk then separates sweet whey from the non-soluble rennet casein and acid whey from acid casein respectively. Finally, soluble milk proteins (ultracentrifuged whey) and non soluble, micellar casein were prepared using ultracentrifugation. All casein and whey fractions were frozen at –20°C until required.

Human mammary cell line

MCF-7 (American Type Culture Center (ATCC), Manassas, VA., HTB-22), a human mammary cell line derived from the pleural effusion of a breast carcinoma, retains several characteristics of differentiated mammary epithelium. The cells were cultured in DMEM (Amimed Bioconcept, Allschwill, Switzerland) supplemented with 10% foetal calf serum (FCS, Amimed Bioconcept) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. The culture media was changed 2 to 3 times per week. Upon reaching confluency, cells were detached using trypsin/EDTA (GibcoBRL) at 37°C. The cells were then prepared for RNA extraction.

Western blot analysis

Milk samples were diluted 1/25 with Laemmli reducing sample buffer and boiled for 5 min. The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (BioRad). The blots were probed with a biotinylated polyclonal anti-human OPG (BAF805 at 0.2 µg/ml; R&D systems) and streptavidin-alkaline phosphatase (Pierce). Immunoreactivity was visualized with alkaline phosphatase substrate BCIP/NBT (Zymed Laboratories). Prestained protein markers were used as molecular weight standards (BioRad). Recombinant human OPG (R&D systems) was load at 25ng/lane served as a positive control.

Expression of OPG by human breast milk cells and human mammary gland epithelial cells

Reverse transcription followed by PCR was used to amplify OPG transcripts in the total human breast milk cell population from a single mother at 18 days postpartum and in the human mammary gland epithelial cell line, MCF-7. Total RNA was extracted from the cells using the Trizol method (Gibco-BRL). Briefly, the Trizol (1 ml for 5–10 x 10^6 cells) was added to the cell pellet, pipetted up and down several times and transferred into an Eppendorf tube. Chloroform was added (0.2 ml for 1 ml Trizol), and the tubes were incubated for 5 min

before centrifugation at 12,000 x g for 15 min, 4 °C. RNA was precipitated with an equal volume of isopropanol and centrifuged at 12,000 x g for 10 min. Pellets were washed with 70% ethanol and then resuspended in sterile, deionized water. RNA was stored at – 20°C until required.

RNA samples were treated with RNase-free DNase I to eliminate contamination by genomic DNA. RNA was quantified by absorbance at 260 nm and 280 nm of an appropriate dilution (100 –200 fold) in a spectrophotometer. The concentration of RNA (μg/ml) was calculated as follow: Absorbance at A_{260} x dilution factor x 40 mg/ml. A total RNA sample that is essentially free of proteins should have an A_{260}/A_{280} ratio of 1.8 – 2.2.

RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer). RNA samples (0.5 μg of total RNA), 0.5 unit of RNase inhibitor, 1 mM of each dNTP, 0.5 nmol/ml of specific 3' primer, 5 mM $MgCl_2$ and 1.25 units of reverse transcriptase were incubated in a total volume of 10 μl of reaction mixture containing the enzyme buffer supplied by the manufacturer. The reaction mixture was incubated for 30 min at 42°C, and then heated for 5 min at 95°C. The reverse-transcripted products were then amplified with Gold DNA polymerase (Perkin Elmer) on a thermocycler (Biolabo, Scientific Instruments, Chatel St Denis, Switzerland). The polymerase chain reaction (PCR) was performed in a total volume of 50 μl using 10 μl of the reverse-transcripted products in PCR buffer, 2 mM $MgCl_2$, 5 μM of each dNTP, 0.2 nmol/ml of both OPG-specific antisense

ACTAGTTATAAGCAGCTTATTTTACTG,

and sense

GGAGGCATTCTTCAGGTTTGCTG

primers and 1.25 units of DNA polymerase. After an initial denaturation step of 10 min at 95°C, samples were amplified by 35 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 1 min, and extension at 72°C of 1 min 30 sec, followed by a 7-min extension step at 72°C. All samples were subjected to RT-PCR with β-actin as a positive control. Samples of RT-PCR products were loaded onto a 1.2 % agarose gels (containing ethidium bromide) in 1 x TAE buffer and separated by electophoresis at 150 V for 1 hr. RT-PCR products were visualised under UV light. The correct size of the bands was determined by comparison with DNA size markers (Boehringer Mannheim).

ELISA for Human OPG-(sandwich enzyme immunoassay)

The concentration of OPG present in breast milk and different milk fractions was measured by ELISA. To this end, monoclonal antibodies against OPG (MAB805, 1 μg/ml; R&D Systems, UK) were coated onto 96-well plates (Nunc) by overnight incubation at 4°C. Plates were then washed twice with 0.05% Tween-20 in PBS. Non-specific binding was blocked by incubating the plates with 2% bovine serum albumin (BSA) in PBS for additional 2 hrs at room temperature. Samples or standard concentrations of recombinant OPG (0.119 to 121.5 ng/ml; R&D Systems) were incubated in PBS-BSA for 3 hrs at room temperature. Plates were then washed four times with PBS-Tween before addition of biotin-labelled anti-human OPG polyclonal antibody (BAF805, 0.5 μg/ml; R&D Systems) for another hour at room temperature. After an additional four washes, streptavidin-peroxidase (SAAP, 0.5 μg/ml. Kirkegaard % Perry KPL) was added for 1 hr at room temperature. Plates were then washed four times, and the substrate TMB peroxidase (KPL) was added. Plates were covered and incubated in the dark for five minutes. The enzymatic reaction was terminated by the addition of 1N HCl. Absorbance was read at 450 nm in an ELISA reader (Dynex Technologies). The detection limit was approximately 30 pg/ml.

Biological activity of human milk OPG

OPG is a receptor for the tumour necrosis factor-related ligand (TRAIL) which induces apoptosis upon binding to its death domain-containing receptors, DR4 and DR5. A bioassay was developed in which human breast milk OPG could be tested for its ability to block the TRAIL-induced apoptosis of these cells.

In this respect Jurkat cells, clone E6-1 (ATCC), were maintained in culture in RPMI 1640 as modified by the supplier ATCC and containing 10% FCS (37°C and 5% $C0_2$). Cells were seeded at a density of 5 x 10^4 cells/well in 96-well plates (Nunc). To each well various concentrations of soluble recombinant human TRAIL (0 to 20 ng/ml) were added in the presence of 2 μg/ml of enhancer protein, an antibody which reacts with soluble recombinant human TRAIL and thereby increases its activity (Alexis, Läufelfingen, CH). Some wells also contained 50 ng/ml recombinant human OPG (R&D Systems), human breast milk samples (HM; 1/80 final dilution; collected at 1d or 9d postpartum) and/or 20 μg/ml anti-OPG monoclonal antibody (MAB805, R&D Systems). Plates were incubated at 37°C for 16 hrs.

Cell viability was measured by adding ^{3}H-thymidine (1 μCi/well) during the last 6 hrs of culture.

In the medium control, a TRAIL-induced inhibition of cell proliferation was evident at concentrations greater than 5ng/ml. However, HM samples prevented this inhibition. This effect was obviously due to the presence of OPG, since the at-OPG monoclonal antibody reversed the effect.

The results are shown in Figure 5.

Western blot analysis

OPG is synthesized as a 55kDa monomer within cells but is converted to a disulphide-linked dimer of approximately 110kDa when secreted extracellularly. In milk bands were detected at approximately 80, 130 and 200 kDa.

Concentrations of OPG in human breast milk

Levels of OPG in the breast milk samples of 10 lactating mothers at different times during the first 17 days of lactation were examined by ELISA. Concentrations increased to maximum values during the first 1-3 days of lactation and then decreased thereafter. Concentrations in milk ranged from 50 ng/ml to almost 2 μg/ml (Figure 1).

Cellular source of milk OPG

RT-PCR analysis revealed that the OPG of the present invention may be found in human breast milk cells and mammary gland epithelial cells. Constitutive expression of mRNA for OPG was evident in both types of untreated cells (Figure 4).

Cloning of human milk OPG in yeast

Cells were isolated from human breast milk (18 days post-partum) by centrifugation (200 x g, 10 min). From the cell pellet, total RNA was extracted using TRIzol® (Life Technologies, Basel, Switzerland), DNAseI treated and further purified on RNeasy spin columns (Qiagen, Basel) as recommended by the manufacturers. A PCR product encoding the mature form of OPG was amplified from this total RNA using the Titan™One tube RT-PCR system following the protocol supplied by the manufacturer (Roche Diagnostics, Rotkreuz).

With the OPG specific antisense primer

CC**GGCCTCTTCGGCC**GCCAAGCGAGAAACGTTTCCTCCAAAGTACC,

and the sense primer

ACTAGTTATAAGCAGCTTATTTTTACTG,

a 1174 bp PCR fragment was amplified from this cDNA. The PCR product was *Sfi*I-*Spe*I digested, gel purified and the resulting 1156 bp fragment ligated to *Sfi*I-*Xba*I digested and SAP-treated pINA1267, creating pNFF270. This plasmid pNFF270 was then introduced into the yeast *Yarrowia lipolytica* by transformation. Figure 3 depicts the restriction map of the plasmid which was integrated into the genomic DNA of *Yarrowia* transformants and SEQ ID. No. 1 the protein encoded by this OPG plasmid.

The sequence of a pGEM-T OPG clone is shown in Figure 6. The mature OPG is in black and translated. In the published OPG/OCIF sequence, amino acid residue 242 of the mature OPG is an Ala-residue (A), whereas all pGEM-T OPG clones analyzed, encoded an Asp-residue (D) at this position. The *Sfi*I-*Spe*I OPG fragment of this clone was transferred to *Sfi*I-*Xba*I digested pINA1267. The resulting plasmid had the restriction map depicted in Figure 3. A single copy of this plasmid was integrated into the genomic DNA of *Yarrowia* transformats. The protein encoded by this plasmid is shown in Figure 4. The mature OPG is indicated in bold print. The plasmid pNFF270 was introduced into *Yarrowia lipolytica* by transformation. The resulting transformants secreted a protein, cross-reacting with OPG-specific antibodies into the culture medium while *Y. lipolytica* transformants carrying the empty expression vector did not secrete such a protein.